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Biological and Biomimetic Materials - Properties to Function

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Self-Assembly of a Modular Polypeptide based on Blocks of Silk-Mimetic and Elastin-Mimetic Sequences

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ABSTRACT

Spider dragline silk fiber displays a unique and technologically significant combination of high tensile and compressive strength. The structural origin of these properties arises from the alternating sequence of crystalline alanine-rich domains and amorphous glycine-rich domains, which undergo microscopic phase separation in the silk fiber. We previously reported the synthesis and the self-assembly of a novel polypeptide **1**, which emulates the modular structure of crystalline and amorphous elastomeric domains in dragline silk proteins. The sequence of this polypeptide comprises an alternating arrangement of a self-complementary, amphiphilic silk-mimetic oligopeptide (Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys) and environmentally-responsive elastin-mimetic segment (Val-Pro-Gly-Val-Gly). We report herein the synthesis and the self-assembly of an analogous polypeptide (**2**) with an higher content of elastin mimetic pentapeptides. A synthetic gene encoding four repeats of the alternating sequence was expressed in *E. coli* strain BL21(DE3) as a C-terminal fusion to a decahistidine leader sequence to afford a polypeptide with a molar mass of approximately 39 kDa. The regularly alternating pattern of elastin-mimetic and silk-mimetic blocks within the protein allowed the copolymer to spontaneously self-assemble upon heating above the phase transition of the elastin-mimetic block. The self-assembly process was studied using a combination of CD and solid-state NMR spectroscopy, which suggested that the alanine-rich domains undergo a conformational rearrangement from α -helix to β -sheet. This rearrangement coincides with the macromolecular phase transition of the elastin-mimetic domains, which resulted in irreversible aggregation of the polypeptide above the T_i of the elastin-mimetic domains.

INTRODUCTION

Spider dragline silk fiber has attracted much scientific interest due to its unique and technologically significant combination of high tensile and compressive strength¹. Dragline silk proteins are composed of alternating sequences of conservatively substituted alanine-rich and glycine-rich oligopeptide segments². Recent spectroscopic^{3,4} and X-ray diffraction⁵ studies of *N. clavipes* dragline fiber suggest that individual chain segments of the silk fibroins segregate into conformationally asymmetric domains and that this mosaic structure underlies the tensile properties of the fiber⁶. The alanine-rich domains adopt a β -strand^{4,7} conformation that may aggregate into either weakly oriented individual sheets or highly oriented crystallites within the fiber. In contrast, the glycine-rich domains form a flexible, amorphous matrix that is responsible for the elasticity of the fiber⁷. The well-defined modular structure of these proteins⁸ suggests potential opportunities for engineering of novel protein polymers based on dragline silk sequences that emulate the properties of natural materials while enhancing the *in vitro* processing characteristics. We have previously reported the design and biosynthesis of a hybrid silk in which the native alanine-rich domain of *A. diadematus* fibroin 3 was replaced with the

amphiphilic oligopeptide sequence [(Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys)₂]⁹. This oligopeptide segment undergoes an irreversible rearrangement from α -helix to β -strand, which drives the self-assembly of the polypeptide into a macroscopic membrane. Later we reported the synthesis and self-assembly of a copolymer incorporating this cross-linkable module (**HB**) into an elastin-mimetic polypeptide sequence (**SB**)¹⁰. We describe herein the synthesis of a analogous block copolymer (**2**) with a molar mass of approximately 39 kDa, using recombinant DNA techniques and bacterial protein expression, that self-assembles through formation of β -sheet segments mediated by the amphiphilic peptides. This polypeptide differs from the previously reported material in that the ratio of elastin-mimetic domains to silk-mimetic domains within the repeat sequence has been increased in order to assess the effect of relative composition on the self-assembly process.

EXPERIMENTAL DETAILS

All chemical reagents were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Corporation (St. Louis, MO) unless otherwise specified. All enzymes were obtained from New England Biolabs, Inc. (Beverly, MA). Plasmid pZErOTM-1.1, *E. coli* strain TOP10F' and ZeocinTM were purchased from Invitrogen Corporation (Carlsbad, CA). Plasmid pET-19b and *E. coli* strain BL21(DE3) were obtained from Novagen (Madison, WI). Synthetic oligonucleotides were from Genosys Biotechnologies, Inc. (The Woodlands, TX). TALON metal affinity resin was purchased from CLONTECH Inc. (Palo Alto, CA). The isotopically labeled amino acid [1-¹³C]alanine was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA).

DNA sequences analyses were performed on a Perkin-Elmer ABI Prism model 377 DNA sequencer. Amino acid compositional analyses were obtained from the Microchemical Facility of the Winship Cancer Center at Emory University. MALDI-TOF mass spectrometry data were performed on an Applied Biosystem Voyager-DETM STR BiospectrometryTM Workstation MALDI-TOF Mass Spectrometer. Protein electrophoresis was performed on 10-15% gradient discontinuous SDS polyacrylamide gels on a PhastSystem from Amersham Pharmacia Biotech and visualized via a silver staining procedure.

Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter. Protein samples were dissolved in sterile water at a concentration of 0.6 mg/mL. Spectra were obtained from 260 nm to 180 nm at a scanning rate of 20 nm/min with a resolution of 0.1 nm and a path length of 0.05 cm.

Solid-state CP/MAS ¹³C spectra were recorded on a Bruker DSX 400 spectrometer (100.6 MHz, ¹³C). Solid-state ¹³C chemical shifts were referenced to the methylene carbons of external adamantane (31.26 ppm) and reported relative to the methyl resonances of an external sample of aqueous 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (0 ppm).

DISCUSSION

Biosynthesis of the protein copolymer

The protein copolymer was biologically synthesized as previously described¹⁰. Briefly, self-ligation of DNA encoding for elastin-mimetic block (Figure 1d) afforded a population of

concatemeric genes, encoding fragments with termini that were compatible with the proximal (Figure 1b) and distal (Figure 1c) **HB** fragments. The concatemers were joined together with the **HB** fragments and inserted into the plasmid pZER0-1.1. A recombinant clone was isolated that encoded three repeats of the **SB** sequence between the **HB** segments, which was verified by DNA sequence analysis. The DNA monomer cassette was excised by restriction digestion with endonuclease *Sap* I. Self-ligation of the monomer afforded a population of concatemers, which were inserted between the *Sap* I sites of a modified polylinker (Figure 1e) introduced into expression plasmid pET-19b. A recombinant clone was isolated that encoded four repeats of the monomer, which was verified by DNA sequence analysis. The plasmid encoding the copolymer gene was introduced into competent cells of *E. coli* strain BL21(DE3). Expression under the induction of 1 mM IPTG in LB medium afforded the target protein as a C-terminal fusion to a short leader sequence containing a decahistidine tag. Electrophoretic analysis of the whole cell lysate as a function of time indicated the gradual accumulation of new protein during a 3 hr induction period. The fusion protein was isolated from the bacterial cell lysate by immobilized metal affinity chromatography and the homogeneity of the protein was confirmed via 10-15% gradient discontinuous SDS polyacrylamide gel electrophoresis. The yield of lyophilized protein is 35 mg per liter cell culture. Amino acid compositional analysis and MALDI-TOF mass spectrometry were consistent with the expected sequence of the protein polymer.



Figure 1. Amino acid sequence of protein 2 (a), and the double stranded DNA sequences of silk-mimetic **HB** blocks (b and c), elastin-mimetic **SB** block (d), and the adaptor gene (e).

Temperature-Dependent Phase Behavior

Elastin-mimetic protein polymers [(Val-Pro-Gly-Xaa-Gly)_n] in aqueous solution display reversible phase separation above a lower critical solution temperature T_i . This process coincides with a conformational rearrangement of the pentapeptide segments from random coil to type II β -turn. The turbidimetric profiles of the protein 2 in aqueous solution at 400 nm (3°C/min) show that the protein is soluble at 25°C, but aggregated as the temperature was raised (Figure 2). The development of turbidity depended on the concentration of 2 as was observed for elastin-mimetic protein polymers (Figure 3)¹¹. A linear dependence between T_{onset} and the logarithm of the concentration was observed, which indicated that the transition involves more than one polypeptide chain¹² (Figure 3). These results suggested that the elastin-mimetic segments of protein polymer 2 undergo conformational rearrangement upon heating above T_i . However, the inverse temperature transition is irreversible since the precipitated polymer does not redissolve at temperatures below T_i . The hydrophobic collapse of the elastin-mimetic blocks above T_i apparently drives an irreversible conformational modification within the silk-mimetic blocks. The overall transition temperature of protein 2 is much higher than that observed for protein 1¹⁰. For example, at a concentration of 1 mg/ml, the onset of phase separation occurs at 38°C for protein 1 but at 50°C for protein 2. This difference may be due to the lower molar mass of 2, which corresponds to a lower total content of elastin-mimetic pentapeptides in comparison to 1 (61 pentapeptides per chain *versus* 111 pentapeptides per chain).

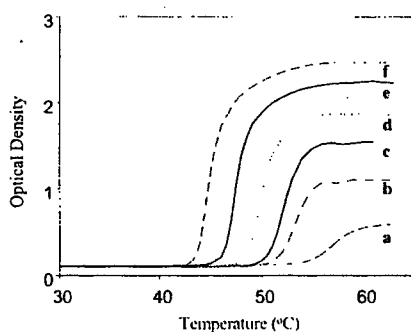


Figure 2. Temperature-dependent turbidity profiles for solutions of protein 2 in water at 400 nm (3°C/min) at different concentration: (a) 0.25 mg/ml, (b) 0.50 mg/ml, (c) 0.66 mg/ml, (d) 1.00 mg/ml, (e) 2.00 mg/ml, (f) 3.40 mg/ml.

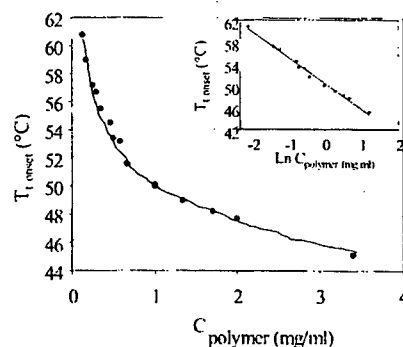


Figure 3. Correlation of the T_{onset} with the concentration of protein 2 in water ($C_{polymer}$ (mg/ml)). Turbidimetry experiments were recorded at 400nm with a scan rate of 3°C/min.

Conformational behavior of polypeptide 2

In situ CD studies were performed on a solution of the protein polymer 2 in water at various temperatures to interrogate the conformational behavior of the polypeptide as the temperature is raised through the phase transition (Figure 4). The CD spectrum initially displayed absorptions that corresponded to those that are typically observed in the spectra of α -helical proteins¹³. As the temperature was raised to 65°C, the CD spectra changed significantly in

a manner that was consistent with formation of β -sheet structure¹⁴. Moreover, specimen did not revert back to the original spectrum upon cooling to ambient temperature, but remained in the β -sheet conformation that was characteristic of the CD spectra at higher temperatures (58°C and 65°C). These data suggest that segments with polypeptide 2 undergo an irreversible α -helix to β -sheet transition that coincides with the phase transition in the elastin-mimetic domains.

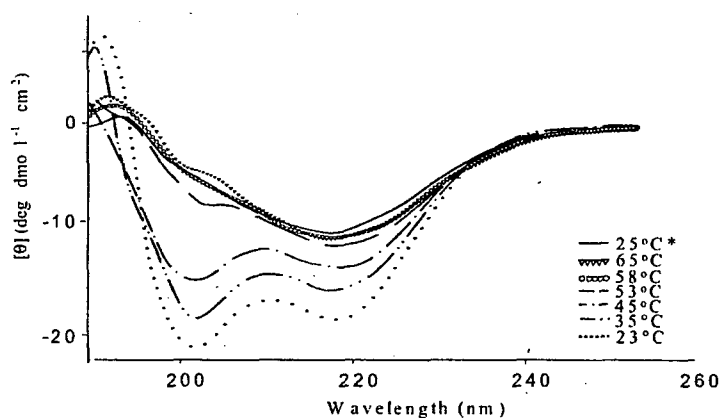


Figure 4. Circular dichroism spectra of protein 2 in water (0.6 mg/ml) as a function of temperature.

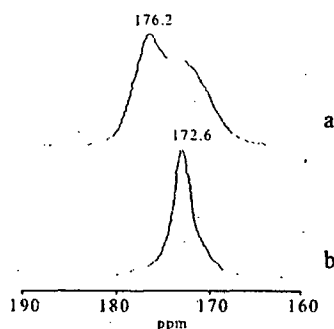


Figure 5. Expansion of the amide carbonyl region within the solid state ^{13}C CP/MAS NMR spectra of lyophilized specimen of 2 (a) and the corresponding heat-treated material (b).

CP/MAS NMR spectroscopy was employed to study the conformation of structural domains within 2 that were site-specifically labeled with NMR-active isotopes. Protein 2 was labeled with the ^{13}C isotope at carbonyl positions of the alanine residues using a procedure similar to that employed for the unlabeled polypeptides. The solid-state ^{13}C NMR spectrum of a nascent specimen of protein 2 revealed a resonance at 176.2 ppm (Figure 5), which is within the expected range for an α -helical conformation for the alanine residues within the silk-mimetic

domains (cf. α -polyalanine, 176.4 ppm)^{14,4}. This result was consistent with the previous CD spectroscopic results, which indicated the presence of α -helices prior to self-assembly. The upfield shoulder of this resonance may reflect the contribution of lyophilization-induced β -sheet formation, which has been observed for similarly treated dragline silk proteins⁴. The ¹³C CP/MAS NMR spectrum of a specimen of protein 2 heated above the phase transition of the elastin-mimetic domain indicated that the alanine carbonyl resonance had shifted upfield to 172.6 ppm, which is within the expected range for a β -sheet conformation (cf. polyalanine 171.8 ppm)¹⁴. These data suggested that the alanine rich block experienced on the α -to- β transition, as consequence of the phase transition of the elastin-mimetic domains.

CONCLUSIONS

A polypeptide multiblock copolymer 2 based on elastin- and silk-mimetic peptide sequences was successfully synthesized via genetic engineering methodology. The elastin-mimetic domains undergo a phase transition in aqueous solution as the temperature is increased. The onset of the phase transition depends on the concentration of the polypeptide. The phase separation of the elastin-mimetic domains induces a conformational transition in the silk-mimetic domains, which results in irreversible aggregation of the polypeptide.

ACKNOWLEDGMENTS

The authors acknowledge the support of NSF grant CHE-9875776.

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